# CTL Fail to Accumulate at Sites of HIV-1 Replication in Lymphoid Tissue[1,2]

**Elizabeth Connick,[3]* Teresa Mattila,† Joy M. Folkvord,* Rick Schlichtemeier,* Amie L. Meditz,* M. Graham Ray,* Martin D. McCarter,‡ Samantha MaWhinney,§ Aaron Hage,† Cara White,† and Pamela J. Skinner†**

**The inability of HIV-1-specific CTL to fully suppress virus replication as well as the failure of administration of exogenous CTL to lower viral loads are not understood. To evaluate the hypothesis that these phenomena are due to a failure of CTL to localize at sites of HIV-1 replication, we assessed the distribution of HIV-1 RNA and HIV-1-specific CTL identified by HIV-1 peptide/HLA class I tetrameric complexes (tetramers) within lymph nodes of 14 HIV-1-infected individuals who were not receiving antiretroviral therapy. A median of 0.04% of follicular compared with 0.001% of extrafollicular $CD4^+$ cells were estimated to be producing HIV-1 RNA, a 40-fold difference ($p = 0.0001$). Tetramer-stained cells were detected by flow cytometry in disaggregated lymph node cells from 11 subjects and constituted a significantly higher fraction of $CD8^+$ cells in lymph node (mean, 2.15%) than in PBMC (mean, 1.52%; $p = 0.02$). In situ tetramer staining in three subjects' lymph nodes, in which high frequencies of tetramer-stained cells were detected, revealed that tetramer-stained cells were primarily concentrated in extrafollicular regions of lymph node and were largely absent within lymphoid follicles. These data confirm that HIV-1-specific CTL are abundant within lymphoid tissues, but fail to accumulate within lymphoid follicles where HIV-1 replication is concentrated, suggesting that lymphoid follicles may be immune-privileged sites. Mechanisms underlying the exclusion of CTL from lymphoid follicles as well as the role of lymphoid follicles in perpetuating other chronic pathogens merit further investigation.** *The Journal of Immunology,* **2007, 178: 6975–6983.**

Human immunodeficiency virus-1-specific $CD8^+$ CTL are thought to play a critical role in controlling HIV-1 replication in vivo. The appearance of HIV-1-specific CTL during acute HIV-1 infection coincides with declines in viremia (1–3), suggesting that CTL may be critical determinants of the initial control of virus replication. Removal of $CD8^+$ cells from SIV-infected macaques leads to increased plasma viremia, further supporting the hypothesis that CTL suppress HIV-1 replication (4, 5). The CTL response to HIV-1 is unique because in some cases virus-specific cytolysis is detectable in PBMC in the absence of in vitro stimulation (6, 7), a phenomenon that has not been frequently reported in other chronic viral infections. Furthermore, high frequencies of HIV-1-specific $CD8^+$ T cells have been detected in PBMC from both acutely and chronically infected individuals using HLA class I tetrameric complexes loaded with HIV-1 epitopic peptides (tetramers) (8–10). Nevertheless, despite evidence that HIV-1-specific CTL are abundant and capable of cytolytic function, they are unable to fully suppress viral replication in vivo resulting in the progressive depletion of $CD4^+$ T cells and, ultimately, death in untreated individuals. Quite perplexingly as well, in multiple studies the administration of exogenous CTL has failed to result in significant decreases in plasma HIV-1 RNA concentrations (11–14). It is not clear why CTL are unable to control HIV infection.

The majority of HIV-1 replication occurs in $CD4^+$ T cells in lymphoid tissues (15, 16). HIV-1 replication is not evenly distributed within lymphoid tissues, but is concentrated within lymphoid follicles where $CD4^+$ cells constitute a minority of cells (17–19). We previously reported that a $CD4^+$ cell within a lymphoid follicle was on average 31-fold more likely to be productively infected compared with a $CD4^+$ cell in the extrafollicular region of lymph node (17). We also demonstrated that multiple antiretroviral effector proteins as well as $CD8^+$ cells are relatively deficient within lymphoid follicles compared with extrafollicular regions of lymph node (17), suggesting that lymphoid follicles may be relatively immune-privileged sites. The existence of an immune-privileged site in lymphoid tissue where HIV-1-specific CTL do not circulate could potentially explain both the inability of endogenous CTL to fully suppress HIV-1 replication as well as the failure of exogenous CTL to decrease viral load. To date, however, the distribution of HIV-1-specific CTL within lymphoid tissues has not been determined, despite their hypothesized importance in controlling HIV-1 replication. The purpose of the present study was to evaluate the magnitude and distribution within lymphoid tissue of HIV-1-specific CTL. We hypothesized that HIV-1-specific CTL would be primarily concentrated in the extrafollicular regions of lymphoid tissues and not in lymphoid follicles where HIV-1 replication is most strongly concentrated.

*Division of Infectious Diseases, University of Colorado at Denver and Health Sciences Center, Denver, CO 80262; †Department of Veterinary and Biomedical Sciences, University of Minnesota, Saint Paul, MN 55108; ‡Department of Surgery, University of Colorado at Denver and Health Sciences Center, Denver, CO 80262; and §Department of Preventive Medicine and Biometrics, University of Colorado at Denver and Health Sciences Center, Denver, CO 80262

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[3] Address correspondence and reprint requests to Dr. Elizabeth Connick, Division of Infectious Diseases, University of Colorado at Denver and Health Sciences Center, Box B168, 4200 East 9th Avenue, Denver, CO 80262. E-mail address: liz.connick@uchsc.edu

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## Materials and Methods

### *Human subjects*

HIV-1-infected individuals who were not receiving antiretroviral therapy were recruited to donate inguinal lymph nodes. None of these subjects had an active opportunistic infection or malignancy or a sexually transmitted disease other than HIV-1. Informed consent was obtained from all participants in accordance with the Colorado Multiple Institutional Review Board.

### *Clinical specimens*

Inguinal lymph node excisional biopsies were performed under local anesthesia at the University of Colorado Hospital as previously described (20). To efficiently identify eligible subjects, before lymph node excision, all subjects were screened for HLA types for which HIV-1 epitope-specific tetramers were available using Abs directed against A2, A3, B7, and B8 (One Lambda). Of note, according to the manufacturer, the B7 Ab also detects individuals with HLA B27 and B42. Only individuals whose cells stained positively with one of these HLA Abs were included in the present study. One portion of the lymph node was placed in RPMI 1640 with sodium heparin (18.7 U/ml) and shipped overnight to the laboratory of Dr. P. Skinner. A second portion of the lymph node was snap-frozen in OCT compound (VWR) and stored at −70°C. A third portion of lymph node was minced in PBS (Invitrogen Life Technologies) to obtain a single-cell suspension and was either used directly in phenotypic or functional assays or was cryopreserved for later studies. Peripheral blood specimens were obtained on the same date and used to determine $CD4^+$ T cell counts by flow cytometry and plasma HIV-1 RNA concentration (HIV-1 Monitor Assay; Roche Diagnostics). PBMC were isolated as well for tetramer staining and functional assays. Subsequent to lymph node excision, all subjects had class I HLA typing performed at the University of Colorado Hospital HLA Typing Laboratory using standard molecular techniques.

### *In situ hybridization for HIV-1 RNA and immunohistochemical staining for CD20 in lymph nodes*

In situ hybridization for HIV-1 RNA was performed on 6-μm tissue sections using digoxigenin-labeled antisense riboprobes for *env*, *gag*, and *nef*, which were detected using NBT/5-bromo-4-chloro-3-indolyl phosphate as previously described (17, 20). This technique identifies cells that are actively transcribing HIV-1, but not extracellular virions encapsulated in envelope glycoprotein. As a negative control, sections from a subset of subjects were hybridized with digoxigenin-labeled sense probes for *env*, *gag*, and *nef* using the same procedure. Immunohistochemical double staining for the B cell marker CD20 was performed as previously described (17) to morphologically identify lymphoid follicles. For each subject, in situ hybridization for HIV-1 RNA and double staining for anti-CD20 was performed in a minimum of three tissue sections, each between 12 and 60 μm apart. The number of virus-producing cells within and outside of lymphoid follicles, defined by anti-CD20 staining, was determined by visual inspection and manually counting the positively stained cells on each section. The area of follicular and extrafollicular lymph node tissue was determined by quantitative image analysis using a computerized image analysis system (Leica Q5001W Image Analysis). The total number of virus-producing cells in follicular and extrafollicular regions was divided by the total area of the follicular or extrafollicular tissue, respectively, to determine the frequency of virus-producing cells per $mm^2$ of follicular and extrafollicular tissue, as previously described (17). The total tissue area evaluated per subject ranged from 53 to 565 $mm^2$, with a median value of 186 $mm^2$.

### *Estimation of frequencies of virus-producing $CD4^+$ cells within lymphoid tissue*

Frequencies of $CD4^+$ cells within lymphoid tissue were estimated by evaluating for each subject three full cross-sections of lymph node, each 60–180 μm apart, that were double stained for CD4 and CD20 as we have previously described (17). Ten randomly selected fields, each 0.74 $mm^2$, were evaluated on each tissue section. Using quantitative image analysis, the area of the follicular and extrafollicular regions was determined as well as the area of each of these regions that stained positively for CD4. The average area of a $CD4^+$ staining cell was determined by measuring the length and width of 10 positively stained cells on each section and using the formula for the area of an ellipse: $\pi \times (\text{mean length}/2) \times (\text{mean width}/2)$. The percentage of the tissue area that stained positively for CD4 was divided by the average area of a $CD4^+$ cell to estimate the number of $CD4^+$ cells per $mm^2$. Frequencies of $CD4^+$ cells that were producing HIV-1 RNA were calculated by dividing the frequency of HIV-1 RNA-producing cells per $mm^2$ by the estimated number of $CD4^+$ cells per $mm^2$.

Table I. *HIV-1 $CD8^+$ T cell epitopes evaluated by tetramer staining*

| HLA Restriction | Epitope Location | Amino Acid Sequence | Abbreviation |
|---|---|---|---|
| HLA-A*0201 | HIV.p17.77–85 | SLYNTVATL | A2 *gag* |
| HLA-A*0201 | HIV.rt.476–484 | ILKEPVHGV | A2 *pol* |
| HLA-A3 | HIV.p17.18–26 | KIRLRPGGK | A3 *gag* |
| HLA-A3 | HIV.*nef*.71–80 | QVPLRPMTYK | A3 *nef* |
| HLA-B7 | HIV.*nef*.128–137 | TPGPGVRYPL | B7 *nef* |
| HLA-B8 | HIV.p24.259–267 | ELYKWKI | B8 *gag* |
| HLA-B8 | HIV.*nef*.89–97 | FLKEKGGL | B8 *nef* |

### *Tetramer staining of PBMC and disaggregated lymph node cells*

PBMC and disaggregated lymph node cells were stained with selected tetramers loaded with $CD8^+$ T cell HIV-1 epitopic peptides (Table I) that matched subjects' HLA class I molecules. Tetramers were selected for analysis based upon prior reports in the literature (21–25) as well as unpublished experience within our laboratories that suggested there was a high likelihood for HIV-1-infected individuals to harbor such cells. Tetramers were obtained from the National Institute of Allergy and Infectious Diseases (NIAID) Tetramer Facility (Emory University, Atlanta, GA), except for those restricted by HLA-A*0201, which were purchased from Beckman Coulter. PBMC were stained with PE- or allophycocyanin-labeled tetramer reagent for 30 min at room temperature with FITC-labeled anti-CD3 Ab and either PE- or allophycocyanin-labeled CD8 Ab (Beckman Coulter) followed by two washes. All samples were analyzed on a FACSCalibur (BD Biosciences) with CellQuest software. Results were expressed as the percentage of tetramer-stained cells in the $CD8^+$ population. Approximately 100,000 gated events were acquired in each analysis. Results were considered positive for tetramer-stained cells if the fraction of tetramer-stained cells that coexpressed CD8 was ≥0.1% of $CD8^+$ cells and a distinct population of cells, characteristic of tetramer staining (10), was observed.

### *In situ tetramer staining*

In situ tetramer staining was performed on fresh lymph node specimens shipped overnight and stained as previously described (26). Biotinylated HLA A2 *gag*, A2 *pol*, or an irrelevant melanoma (FLAGHIEIVPV) peptides were synthesized at Beckman Coulter Immunomics. Biotinylated HLA A3 *gag*, A3 *nef*, B8 *gag*, B8 *nef*, and B7 *nef* tetramers were produced at the NIAID Tetramer Facility. HLA/peptide tetramers were produced by adding six aliquots of FITC-labeled ExtraAvidin (Sigma-Aldrich) to biotinylated HLA/$\beta_2$-microglobulin/peptide monomers over the course of 8 h to a final molar ratio of 4.5:1. Fresh lymph node tissues were cut into 0.5-cm pieces and embedded in 4% low melt agarose. Tissue blocks were placed in a vibratome bath containing 0–2°C PBS supplemented with heparin (100 μg/ml; Sigma-Aldrich) (PBS-H),[4] and 200-μm thick sections were generated. Fresh sections were stained free floating in 1 ml of solution with one to four sections per well in 24-well tissue culture plates. Incubations were conducted at 4°C on a rocking platform. Tetramers at a concentration of 0.5 μg/ml with 2% normal goat serum (NGS) and 0.5 μg/ml mouse anti-human CD8 Abs (DakoCytomation; clone DK25) or anti-human CD20 Abs (Nova Castra; clone L26) were diluted in PBS-H and incubated overnight. Sections were washed with chilled PBS-H and then fixed with freshly made 4% paraformaldehyde for 2 h at room temperature. Sections were again washed with PBS-H and then incubated with rabbit anti-FITC Abs (BioDesign) diluted 1/10,000 in PBS-H with 2% NGS and incubated 1–3 days. Sections were washed three times with PBS-H for at least 20 min and then incubated with Cy3-conjugated goat anti-rabbit Abs and Alexa 488-conjugated goat anti-mouse Abs (Jackson ImmunoResearch Laboratories) both diluted 1/1,000 in PBS-H with 2% NGS for 1–3 days. Finally, sections were washed three times for at least 20 min in PBS-H, postfixed with 4% paraformaldehyde for 1 h, and then mounted on slides with warmed glycerol gelatin (Sigma-Aldrich) containing 4 mg/ml *n*-propyl gallate (Fluka). Stained sections were analyzed using a Bio-Rad 1000 confocal microscope. The individual who analyzed in situ tetramer-stained specimens was blinded to the results of tetramer staining on PBMC and disaggregated lymph node cells.

[4] Abbreviations used in this paper: PBS-H, PBS supplemented with heparin; NGS, normal goat serum; B-LCL, B-lymphoblastoid cell line; CI, confidence interval; KSHV, Kaposi's sarcoma herpes virus.

Table II. *Demographic and clinical characteristics of study subjects*

| Subject ID | Age (years) | Sex | Plasma HIV-1 RNA ($log_{10}$ copies/ml) | $CD4^+$ T Cells/mm$^3$ | HLA A and B Alleles | |
|---|---|---|---|---|---|---|
| 32 | 48 | Female | 3.71 | 775 | A0301, 1101 | B0702, 3501 |
| 61[a] | 42 | Male | 3.29 | 821 | *A0301, 2902* | *B1402, 4501* |
| 62[a] | 32 | Male | 4.35 | 305 | *A0201, -* | *B0702, 1524* |
| 64 | 25 | Female | 3.44 | 467 | *A0201, -* | *B2705, 5101* |
| 67[a] | 45 | Female | 4.37 | 406 | *A0201, -* | *B2705, 5101* |
| 79[a] | 42 | Female | 4.74 | 653 | *A0201, 0301* | *B1402, 3906* |
| 80 | 43 | Male | 3.94 | 628 | *A2402, 2902* | *B0801, 5801* |
| 81 | 29 | Male | 4.23 | 697 | *A0101, 0206* | *B0801, 3906* |
| 82 | 41 | Female | 3.57 | 835 | *A0201, 0205* | *B1501, 5301* |
| 84 | 39 | Female | 3.83 | 358 | *A3101, 3201* | *B1501, 3801* |
| 86 | 31 | Female | 4.01 | 460 | *A0301, 6601* | *B0702, 5802* |
| 87[a] | 26 | Female | 4.36 | 256 | *A0201, 3002* | *B1801, 3901* |
| 90 | 34 | Female | 4.86 | 332 | *A2301, 2601* | *B0801, 5802* |
| 92 | 40 | Female | 4.02 | 671 | *A0201, 0301* | *B2705, 4101* |

[a] Subjects who previously had received antiretroviral therapy. $CD4^+$ T cell counts indicated in the table were also the nadir $CD4^+$ T cell counts for subjects 62, 79, and 87. Nadir $CD4^+$ T cell counts were 636 cells/mm$^3$ for subject 61 and 398 cells/mm$^3$ for subject 67.

*Assays for HIV-1-specific cytolytic activity*

Freshly isolated lymph node cells and PBMC were assayed for HIV-1-specific cytolytic activity in $^{51}Cr$ release assays as we have previously described (2), using as target cells autologous B-lymphoblastoid cell lines (B-LCL) pulsed with HIV-1 $CD8^+$ T cell epitopic peptides. B-LCL were established by EBV transformation of PBMC (7). For selected subjects known to harbor tetramer-stained cells before lymph node excision, B-LCL were labeled with $^{51}Cr$ and incubated for 1 h with the appropriate HIV-1 epitopic peptide (100 $\mu$g/ml) (Table I) synthesized by Multiple Peptide Systems. As a control for A2-restricted peptides, $^{51}Cr$-labeled B-LCL were incubated with the irrelevant A2-restricted melanoma peptide. For HIV-1 peptides restricted by other HLA molecules, $^{51}Cr$-labeled B-LCL alone were used as a control. Freshly isolated lymph node cells or PBMC were incubated for 6 h with HIV-1 peptide-pulsed $^{51}Cr$-labeled B-LCL or control B-LCL, at E:T cell ratios of 200:1 (if sufficient cells were available), 100:1, 50:1, and 25:1. Maximum $^{51}Cr$ release was determined by detergent lysis of target cells with 5% Triton X-100 and spontaneous release was determined by incubating target cells in the absence of effector cells. Supernatants (0.05 ml) were harvested and the number of cpm was determined on a $\gamma$ counter (Beckman Instruments). Percent cytotoxicity for peptide-pulsed B-LCL and control B-LCL was determined as: (mean observed cpm − mean spontaneous cpm)/(mean maximum cpm − mean spontaneous cpm) (2). The tetramer stained cell-to-target cell ratio was determined by multiplying the E:T cell ratio by the percentage of lymph node cells or PBMC that stained with tetramers as determined by flow cytometry.

*Statistical analysis*

All statistical analyses assumed a two-sided significance level of 0.05. Analyses were conducted using SAS (SAS Institute) and Splus (Insightful Corporation) software. Reported mean values for frequencies of HIV-1 RNA-producing cells per mm$^2$ by lymph node region were obtained using Poisson regression with generalized estimating equations accounting for within-subject correlation. For this regression, the count of HIV-1 RNA-producing cells within the lymph node region was the dependent variable with the corresponding $\log_e$ area included as an offset. Tetramer analyses used generalized least squares regression to account for within-subject correlations. Before analysis, data were plotted to ascertain normality. Correlations were conducted using Spearman $\rho$.

## Results

*Demographic and clinical characteristics of study subjects*

Fourteen HIV-1-infected individuals screened positively with Abs directed at selected HLA molecules and were included in this study. Demographic and clinical characteristics of these individuals as well as their *HLA A* and *B* alleles as determined by molecular HLA typing are shown in Table II. Subject 64 was recruited 4 mo after documented primary HIV-1 infection. All other subjects had been infected for more than 1 year. None were receiving antiretroviral therapy at the time of the lymph node excision and the majority were antiretroviral therapy naive. Five subjects had received some form of antiretroviral therapy in the past, but had discontinued therapy a median of 2 years (range, 8 mo to 5 years) before lymph node excision. The median $CD4^+$ T cell count was 467 cells/mm$^3$ and the median plasma HIV-1 RNA concentration was 4.01 $\log_{10}$ copies/ml. None of the subjects had a history of an opportunistic infection or a $CD4^+$ T cell nadir below 250 cells/mm$^3$.

*Frequencies of HIV-1 RNA-producing cells detected in lymph node*

To characterize the distribution of HIV-1-producing cells within lymphoid tissue, we performed in situ hybridization on frozen lymph node sections using antisense riboprobes for *env*, *gag*, and *nef*. Frequencies of HIV-1 RNA-producing cells per mm$^2$ lymph node tissue ranged from 0.05 to 0.52 with a median of 0.21. Control sections treated with sense probes were uniformly negative. Frequencies of HIV-1 RNA-producing cells within lymph node correlated with plasma HIV-1 RNA concentration (Spearman $\rho$ = 0.59; $p$ = 0.035), suggesting that these cells are representative of the major virus-producing cells in the body. A median of 17% (range, 3–50%) of subjects' lymph node tissue consisted of lymphoid follicles, defined morphologically by anti-CD20 staining (17). A median of 60% (range, 29–87%) of HIV-1 RNA-producing cells as determined by in situ hybridization were located within lymphoid follicles. Frequencies of HIV-1 RNA-producing cells per mm$^2$ tissue were uniformly higher within lymphoid follicles compared with extrafollicular regions, as previously reported by our group (17). A mean of 0.69 (95% confidence interval (CI): 0.55, 0.87) HIV-1 RNA-producing cells per mm$^2$ tissue were detected within lymphoid follicles compared with 0.09 (95% CI: 0.05, 0.16) cells per mm$^2$ in extrafollicular regions of lymph node ($p < 0.0001$).

The number of $CD4^+$ cells per mm$^2$ lymph node tissue was calculated based upon immunohistochemical staining and estimates in each subject of the average area of a $CD4^+$ staining cell (Table III). The median density of $CD4^+$ cells in follicular regions was 21% (range, 15–50%) of that in extrafollicular regions of lymphoid tissue, and is consistent with the known anatomy of lymph nodes as well as previous reports from our laboratory (17). Based on prior observations that the vast majority of HIV-1-producing cells in lymphoid tissues in vivo are $CD4^+$ cells (15, 16, 27), the

Table III. *Estimated densities of $CD4^+$ cells and frequencies of HIV-1-producing $CD4^+$ cells in lymph nodes of HIV-1-infected subjects ordered by ascending plasma viral load*

| Subject ID | Estimated Number of $CD4^+$ Cells/mm$^2$[a] | | | Estimated Number of HIV-1-Producing Cells/$10^6$ $CD4^+$ Cells[b] | | |
|---|---|---|---|---|---|---|
| | Lymph node | Inside follicle | Outside follicle | Lymph node | Inside follicle | Outside follicle |
| 61 | 13,890 | 3,685 | 16,310 | 16 | 594 | 3 |
| 64 | 7,335 | 1,470 | 9,713 | 12 | 382 | 2 |
| 82 | 6,254 | 1,558 | 7,350 | 24 | 391 | 7 |
| 32 | 7,825 | 1,769 | 9,822 | 13 | 220 | 1 |
| 84 | 5,476 | 1,171 | 6,333 | 13 | 123 | 8 |
| 80 | 5,739 | 1,524 | 6,995 | 35 | 286 | 14 |
| 86 | 2,563 | 1,379 | 2,766 | 123 | 414 | 79 |
| 92 | 9,170 | 2,054 | 11,185 | 17 | 307 | 8 |
| 81 | 4,408 | 1,340 | 4,973 | 104 | 1,644 | 40 |
| 62 | 10,304 | 4,180 | 11,602 | 5 | 261 | 2 |
| 87 | 5,178 | 1,414 | 6,468 | 101 | 590 | 33 |
| 67 | 8,085 | 2,282 | 10,019 | 44 | 617 | 16 |
| 79 | 3,999 | 1,009 | 5,052 | 120 | 1,265 | 50 |
| 90 | 3,822 | 1,127 | 4,575 | 84 | 922 | 30 |
| Median | 5,997 | 1,497 | 7,173 | 30 | 403 | 11 |

[a] The number of $CD4^+$ cells per mm$^2$ tissue was calculated by dividing the percentage of the tissue area that stained positively for CD4 by the average area of a $CD4^+$ staining cell in the same tissue section. Differences between follicular and extrafollicular tissues were statistically significant ($p = 0.0001$).

[b] The number of HIV-1-producing $CD4^+$ T cells per $10^6$ $CD4^+$ cells was determined by dividing the frequency of HIV-1-producing cells per mm$^2$ tissue as determined by in situ hybridization by the estimated number of $CD4^+$ T cells per mm$^2$ tissue. Differences between follicular and extrafollicular tissues were statistically significant ($p = 0.0001$).

frequency of HIV-1-producing $CD4^+$ cells in follicular and extrafollicular regions of lymph node was estimated for each subject by combining the results from in situ hybridization for HIV-1 RNA with the results from the $CD4^+$ cell quantification (Table III). A median of 30 per $10^6$ or 0.003% of lymph node $CD4^+$ cells were estimated to be producing HIV-1 RNA. Frequencies of infected $CD4^+$ cells were ~40 times higher in lymphoid follicles (median, 0.04% of $CD4^+$ cells) than in extrafollicular regions (median, 0.001% of $CD4^+$ cells; $p = 0.0001$).

*Tetramer staining of PBMC and disaggregated lymph node cells*

To characterize HIV-1-specific CTL in PBMC and disaggregated lymph node cells, flow cytometric assays were performed using 7 HIV-1 tetramers restricted by four different HLA molecules. A total of 37 tetramer-staining assays were performed on paired PBMC and disaggregated lymph node cells in the 14 subjects recruited to this study (Table IV). Tetramer-stained cells were detected in 15 PBMC assays and 16 disaggregated lymph node cell assays, and these were concordant in terms of the presence or absence of tetramer-stained cells in all instances in which results were available for both compartments. Tetramer-stained cells were detected by all tetramers used except for the A3 *gag* tetramer. Tetramer-stained cells were detected by the A3 *gag* tetramer and confirmed in functional assays in a patient not included in the present study (data not shown), demonstrating that the tetramer was not defective. Neither plasma HIV-1 RNA concentration nor frequencies of HIV-1 RNA-producing cells in lymph node were a significant predictor of percentage of lymph node tetramer-stained cells ($p = 0.84$ and $p = 0.31$, respectively). The mean percentage of lymph node $CD8^+$ cells that stained positively with tetramers was 2.15% (95% CI: 1.20, 3.10) and was significantly higher than that in PBMC (mean, 1.52%; 95% CI: 0.54, 2.50) with a mean difference of 0.70% (95% CI: 0.16, 1.23; $p = 0.02$).

Table IV. *Tetramer staining of PBMC, disaggregated lymph node cells, and lymph node sections*[a]

| Subject ID | Epitope | % Tetramer$^+$/$CD8^+$ Cells | | In Situ Tetramer Staining |
|---|---|---|---|---|
| | | PBMC | Lymph node | |
| **32** | A3/*gag* | Negative | Negative | Negative |
| | A3/*nef* | Negative | Negative | nd |
| | B7/*nef* | nd[b] | **0.2** | Negative |
| 61 | A3/*gag* | Negative | Negative | Negative |
| | A3/*nef* | Negative | Negative | Negative |
| 62 | A2/*gag* | **4.1** | **5.3** | **Positive** |
| | A2/*pol* | Negative | Negative | Negative |
| 64 | A2/*gag* | **2.7** | **4.8** | **Positive** |
| | A2/*pol* | Negative | Negative | Negative |
| 67 | A2/*gag* | Negative | Negative | Negative |
| | A2/*pol* | Negative | Negative | Negative |
| | B7/*nef*[c] | **0.4** | **1.9** | **Positive** |
| 79 | A2/*gag* | **0.4** | **1.2** | **Positive** |
| | A2/*pol* | **0.1** | **0.4** | nd |
| | A3/*gag* | Negative | Negative | Negative |
| | A3/*nef* | Negative | Negative | Negative |
| 80 | B8/*gag* | **0.4** | **1.4** | nd |
| | B8/*nef* | Negative | Negative | Negative |
| 81 | B7/*nef*[c] | Negative | Negative | Negative |
| | B8/*gag* | **0.8** | **1.0** | **Positive** |
| | B8/*nef* | **1.9** | **3.2** | **Positive** |
| 82 | A2/*gag* | Negative | Negative | Negative |
| | A2/*pol* | **0.4** | **0.8** | Negative |
| 84 | B7/*nef*[c] | Negative | Negative | Negative |
| 86 | A3/*gag* | Negative | Negative | Negative |
| | A3/*nef* | **2.6** | **3.6** | nd |
| | B7/*nef* | **1.0** | **2.1** | **Positive** |
| 87 | A2/*gag* | Negative | Negative | Negative |
| | A2/*pol* | Negative | Negative | Negative |
| 90 | B7/*nef*[c] | Negative | Negative | nd |
| | B8/*gag* | **0.7** | **0.7** | nd |
| | B8/*nef* | **7.0** | **5.1** | nd |
| 92 | A2/*gag* | Negative | Negative | Negative |
| | A2/*pol* | **0.2** | **0.4** | Negative |
| | A3/*gag* | Negative | Negative | Negative |
| | A3/*nef* | **0.1** | **0.2** | nd |
| | B7/*nef*[c] | Negative | Negative | Negative |

[a] Positive responses are highlighted in bold type.

[b] nd indicates that an assay was not done due to insufficient amounts of tissues or cells, or shipping problems.

[c] Subject was tested for B7/*nef*-specific cells because screening Ab tests with the B7 Ab were positive, but HLA B7 was not confirmed on molecular HLA typing (Table II).

*In situ tetramer staining of lymph nodes*

To determine the spatial localization of HIV-1-specific $CD8^+$ T cells in lymph nodes of HIV-1-infected subjects, in situ tetramer staining was performed on fresh lymph node sections. HIV-1-specific cells were detected within intact lymph node specimens by in situ tetramer staining in 7 of 13 subjects analyzed (Table IV). In situ tetramer-stained sections that were counterstained with CD8 Abs showed that most tetramer-stained cells coexpressed CD8 (Fig. 1, *A–C*). Negative controls for the in situ tetramer staining included sections stained with tetramers loaded with irrelevant peptides and HLA-mismatched patient tissue stained with tetramers loaded with HIV peptides, and results were similar to that seen in tissues from patients that did not show detectable tetramer staining (Fig. 1, *D–F*). Positive results from in situ tetramer staining were confirmed in all instances by tetramer staining of disaggregated lymph node cells (Table IV). In three instances, tetramer-stained cells were detected in disaggregated lymph node cells, but in situ studies did not demonstrate tetramer staining above background levels. The percentages of tetramer-stained cells in these

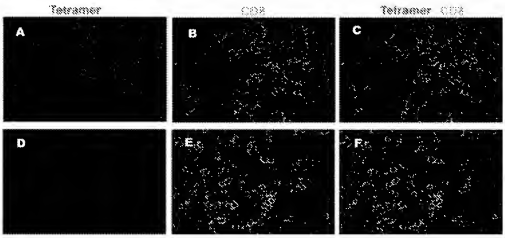


**FIGURE 1.** HLA class I tetramer (red; *left panels*) and CD8 (green; *middle panels*) staining and merged images (*right panels*) in lymph node sections from two HIV-1-infected individuals. Lymph node from subject 62 (*A–C*) stained with A2 *gag* tetramer and CD8 Ab demonstrated coexpression of CD8 by the majority of tetramer-stained cells, as shown in the merged image (*C*). Lymph node from subject 67 (*D–F*) stained with A2 *gag* tetramer and CD8 Ab demonstrated no evidence of tetramer-stained cells, although $CD8^+$ cells were present. Images are confocal Z-scans collected 9 $\mu$m into the tissue sections with a ×60 objective using the same confocal parameters.

three cases were <1% of $CD8^+$ cells, suggesting that in situ tetramer staining is less sensitive than flow cytometric analysis of disaggregated lymph node cells.

To characterize the distribution of HIV-1-specific CTL within and outside of lymphoid follicles, double staining with anti-CD20 Abs and tetramers was performed. Tissues from three subjects showed abundant tetramer-stained cells as well as multiple follicles within stained tissue sections and were used for this analysis. Subjects 62 and 64 harbored tetramer-stained cells against an HLA A*0201-restricted *gag* epitope, whereas subject 81 harbored tetramer-stained cells against an HLA B8-restricted *nef* epitope. Substantially, fewer tetramer-stained cells were localized inside follicles compared with extrafollicular regions in lymph node sections from these subjects (Fig. 2). Many follicles were almost entirely devoid of any tetramer-stained cells (Fig. 2, *A–C*). In follicles that showed some tetramer-stained cells, the tetramer-stained cells frequently localized near one edge of the follicle with the remaining areas of the follicle devoid of tetramer-stained cells (Fig. 2, *D–F*).

### *HIV-1 epitope-specific cytolytic activity mediated by tetramer-stained cells*

To evaluate the cytolytic function of tetramer-stained cells, HIV-1 epitope-specific cytotoxicity was assessed in $^{51}Cr$ release assays using PBMC and disaggregated lymph node cells and autologous B-LCL pulsed with HIV-1 peptides (Fig. 3). These assays were performed in three subjects known to harbor tetramer stained cells and for whom autologous B-LCL were available at the time of lymph node excision. Subjects 62 and 64 both had tetramer-stained cells directed against the A2 *gag* epitope, and subject 67 had tetramer-stained cells directed against the B7-restricted *nef* epitope (Table IV). Controls for these assays consisted of autologous B-LCL pulsed with an irrelevant A2-restricted melanoma peptide for

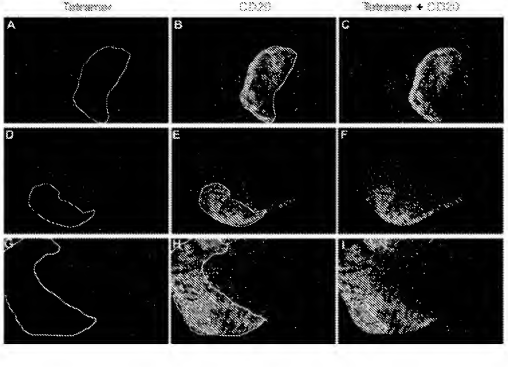


**FIGURE 2.** HLA class I tetramer (red; *left panels*) and CD20 (green; *middle panels*) staining and merged images (*right panels*) in representative lymph node sections from three HIV-1-infected individuals. Lymphoid follicles, defined morphologically by CD20 staining, are outlined in white. Lymph node sections from subject 64 (*A–C*) and subject 62 (*D–F*) were stained with A2 *gag* tetramer and lymph node sections from subject 81 (*G–I*) were stained with B8 *nef* tetramer. Lymph node sections from each subject showed markedly fewer tetramer-stained cells localized within follicles compared with extrafollicular regions. Images are confocal Z-scans collected using a ×10 objective.

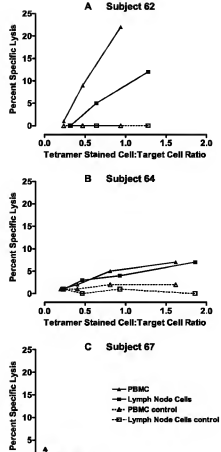


**FIGURE 3.** Cytolysis mediated by PBMC (triangles) and lymph node cells (squares) against B-LCL pulsed with epitopic peptides (closed symbols) or controls (open symbols) from three HIV-1-infected subjects. PBMC and lymph node cells from subjects 62 (*A*) and 64 (*B*) demonstrated concentration-dependent cytolysis against autologous B-LCL pulsed with an A2-restricted *gag* peptide compared with autologous B-LCL pulsed with an irrelevant peptide. Neither PBMC nor lymph node cells from subject 67 (*C*) demonstrated cytolysis against a B7-restricted *nef* peptide compared with autologous B-LCL alone, although tetramer-to-target cell ratios tested were much lower in this subject than in the previous two subjects.

the A2 *gag* assays, or B-LCL alone for the B7 *nef* assays. As shown in Fig. 3, *A* and *B*, concentration-dependent cytolytic activity was mediated by the PBMC and lymph node cells of both subjects 62 and 64 against autologous B-LCL pulsed with the HIV *gag* peptide at tetramer-to-target cell ratios between 1:1 and 2:1, but not against autologous B-LCL pulsed with an irrelevant melanoma peptide. Subject 67 (Fig. 3*C*) did not demonstrate any significant cytolysis of either HIV-1 peptide-pulsed target cells or control cells. However, in subject 67 the tetramer-stained cell-to-target cell ratios were all $<1:1$, and therefore too low to determine whether these cells were capable of mediating cytolytic activity comparable to the tetramer-stained cells of the other two subjects. Importantly, in vitro studies of HIV-1-specific CTL clones usually use substantially higher E:T cell ratios, often as much as 10:1 or 20:1 to detect cytolytic activity (2, 28). Thus, cytolytic activity was detected in lymph node cells at low E:T cell ratios in the present study and there was no evidence of gross cytolytic dysfunction.

## Discussion

The inability of HIV-1-specific CTL to fully suppress virus replication as well as the failure of administration of exogenous CTL to produce declines in plasma viral load have never been adequately explained. We evaluated the magnitude and distribution of HIV-1-specific CTL detected by tetramers within lymph nodes of untreated, chronically HIV-1-infected individuals to assess the hypothesis that HIV-1-specific CTL fail to localize at sites of virus replication, which could explain these perplexing findings. The present study confirms previous observations that HIV-1 replication is concentrated primarily within lymphoid follicles (17–19, 29, 30) and provides the first estimates of the percentage of $CD4^+$ cells that are productively infected within these two compartments. Tetramer-stained cells were abundant, constituting >5% of lymph node $CD8^+$ cells in some cases, and were a significantly larger fraction of lymph node than PBMC $CD8^+$ cells. In situ analyses revealed that tetramer-stained cells failed to accumulate within lymphoid follicles where virus replication was concentrated, but were located primarily in the extrafollicular regions of lymph node. These findings confirm and extend our previous observations that multiple antiretroviral effector proteins are relatively deficient within lymphoid follicles compared with extrafollicular regions of lymph node (17) and lend credence to the hypothesis that a deficiency in immune responses in lymphoid follicles promotes virus replication at those sites.

Between 0.0005 and 0.01% of lymph node $CD4^+$ cells were estimated to be productively infected in the present study. These estimates were based upon prior observations that the vast majority of productively infected cells within lymphoid tissues during asymptomatic HIV-1 infection are $CD4^+$ cells (27) and are similar to estimates previously reported in the literature for individuals at this stage of disease (31, 32). A median of 0.04% of follicular $CD4^+$ cells were estimated to harbor HIV-1 RNA, ~40-fold more than the percentage of HIV-1 RNA-producing $CD4^+$ cells in extrafollicular tissues. To our knowledge, this is the first study to quantify the percentage of productively infected $CD4^+$ cells within lymphoid tissue subcompartments and further strengthens the observation that HIV-1 replication is concentrated primarily within lymphoid follicles of lymphoid tissues (17–19, 29, 30). A previous study found that 59% of the HIV-1 RNA-producing cells in lymphoid tissues in early stages of HIV-1 infection express HLA-DR and 43% express Ki67 (33), which are markers of cellular activation and proliferation, respectively. The phenotype of follicular $CD4^+$ cells that are replicating HIV-1 has never been reported. It is probable, however, that a significant fraction of productively infected follicular $CD4^+$ cells express Ki67 and HLA-DR, because the majority of HIV-1-producing cells are within lymphoid follicles and follicular dendritic cells previously have been shown to induce activation and proliferation of $CD4^+$ T cells (34). The presence of large amounts of highly infectious HIV-1 virions on the surface of follicular dendritic cells (31, 35), as well as the ability of follicular dendritic cells to activate and induce proliferation of $CD4^+$ T cells are likely key determinants of the concentration of HIV-1 RNA production within lymphoid follicles.

We demonstrated in the present study that tetramer-stained cells were more concentrated in lymph node than peripheral blood, consistent with a previous study that observed higher concentrations of HIV-1 tetramer-stained cells in gut-associated lymphoid tissues than PBMC (36, 37). However, another earlier study did not detect significant differences between concentrations of tetramer-stained cells in PBMC and peripheral lymph node cells (24). It is unclear whether the discrepancy in results between the previous lymph

node study and the present one relates to technical differences in how tetramer-stained cells were measured, or whether there were clinical differences among subjects. The present study, nonetheless, adds to the literature demonstrating that HIV-1-specific $CD8^+$ cells readily accumulate within lymphoid tissues of HIV-1-infected individuals and provides persuasive evidence against the hypothesis that HIV-1-specific cells are selectively excluded from lymphoid tissues (38).

It has been suggested that ongoing virus replication in untreated HIV-1-infected individuals is due to dysfunction of virus-specific CTL (39). Virus-specific IFN-γ responses do not consistently reflect cytolytic capabilities (40) and HIV-1-specific tetramer-stained cells demonstrate impairments of cytokine release (41) and cytolytic function (25). Furthermore, a failure of perforin to be coexpressed with granzyme A within lymphoid tissues of HIV-1-infected individuals has been interpreted as evidence of a cytolytic defect (42). In the present study, we evaluated HIV-1-specific activity using conventional $^{51}Cr$ release assays rather than IFN-γ responses or immunohistochemical staining for perforin and granzyme A because, although $^{51}Cr$ release assays are substantially less sensitive, they definitively demonstrate cytolytic capability. We observed epitope-specific, concentration-dependent cytolytic activity in lymph node cells and PBMC from two of three subjects assayed at quite low tetramer-to-target cell ratios. These data are consistent with a previous report of cytolytic activity mediated by spleen and tonsil cells from two HIV-1-infected individuals (43). Collectively, these data do not support the hypothesis that HIV-1-specific $CD8^+$ T cells in lymphoid tissues are grossly impaired in terms of cytolytic function.

Ours is the first study to characterize the distribution of endogenous HIV-1-specific CTL within lymphoid tissues. We found that HIV-1-specific CTL localized primarily in extrafollicular regions of lymph node, away from sites of virus replication, in all three subjects analyzed. A previous study that evaluated the spatial localization within lymph nodes of gene-marked CTL infused into three HIV-1-seropositive subjects also found that they localized primarily in extrafollicular regions (44), suggesting that trafficking of gene-marked CTL is similar to that of endogenous CTL. Although limited numbers of subjects were evaluated, the consistency of this finding in both studies strengthens the conclusion that HIV-1-specific CTL localize within lymphoid tissues, but fail to accumulate within lymphoid follicles.

The interaction of chemokines with their cognate receptors is thought to be the major determinant of localization of cells within lymphoid tissues resulting in distinct microenvironments. The chemokine receptor CCR7, which is found primarily on naive and central memory T lymphocytes (45), induces the migration of these cells from peripheral blood into lymph node and promotes their localization within the extrafollicular or T cell zones of lymph node where the CCR7 ligands, CCL19 and CCL21, are concentrated. Follicular $CD4^+$ T cells constitute a specialized subset of Th cells that provide critical signals to B cells through costimulatory molecules and cytokines that induce Ab class switching and proliferation (46, 47). Migration of this specialized subset of $CD4^+$ T cells into lymphoid follicles requires both up-regulation of CXCR5, the receptor for the B cell-attracting chemokine 1 (CXCL13), as well as down-regulation of CCR7 (48). Importantly, few $CD8^+$ T cells express CXCR5, suggesting differential regulation of this receptor in T cell subsets (48). We previously reported that $CD8^+$ T cells are primarily concentrated within extrafollicular regions of lymph node in both HIV-1-seropositive and -seronegative individuals (17) and limited CXCR5 expression on $CD8^+$ cells may explain this. Teleologically, it makes sense that lymphoid follicles might exclude $CD8^+$ T cells, because they could interfere with the process of affinity maturation of Ab-producing cells. Thus, the failure of HIV-1-specific $CD8^+$ T cells to localize within lymphoid tissues likely results from the normal biology of the lymphoid tissue microenvironment and not a specific derangement induced by HIV-1.

The finding that HIV-1 replication is concentrated within lymphoid follicles, but that virus-specific CTL do not accumulate at those sites, indicates that lymphoid follicles are immune-privileged sites. The model of immune privilege within lymphoid follicles suggested by the findings of this study helps to explain why untreated individuals demonstrate ongoing HIV-1 replication despite large numbers of functional HIV-1-specific CTL. It also explains the paradox that depletion of $CD8^+$ T cells results in dramatic increases in plasma viral load (4, 5), but administration of exogenous CTL fails to produce significant declines in viral load (11–14), a paradox that cannot be explained by immune dysfunction. According to the model proposed here, endogenous CTL can never fully suppress HIV-1 replication because they do not accumulate in lymphoid follicles where HIV-1 replication is focused. Depletion of $CD8^+$ T cells from lymphoid tissue allows virus replication to spread to extrafollicular areas where the majority of $CD4^+$ T cells are located resulting in increased viral loads. The administration of exogenous CTL, however, fails to produce significant declines in virus replication because exogenous CTL are likewise unable to accumulate in lymphoid follicles and endogenous CTL are already highly effective in suppressing HIV-1 replication in the extrafollicular regions. This paradigm also explains why HIV-1-specific $CD8^+$ T cell responses do not correlate inversely with plasma viral load in the present study and other studies of lymphoid tissues (36, 37, 49–51), as well as the vast majority of studies of peripheral blood (6, 8, 52–55). In this model, the correlates of plasma HIV-1 RNA concentration would be factors that are present within the follicular milieu, such as chemokine receptor expression on host $CD4^+$ T cells (56) and virus replicative capacity (57), as has been observed. Because HIV-1-specific $CD8^+$ T cells are largely excluded from follicles, neither their magnitude nor their breadth would be expected to correlate with plasma HIV-1 concentrations and, by inference, to disease progression.

Several directions for future research are suggested by the finding that HIV-1-specific CTL fail to accumulate within lymphoid follicles. First, the most convincing evidence that lymphoid follicles evade immune surveillance by host CTL would be to demonstrate that redistribution of CTL to lymphoid follicles results in improved virus suppression. To accomplish this, it is essential to better understand the mechanisms that might induce migration of CTL into follicles. The failure of HIV-1-specific $CD8^+$ T cells to accumulate within lymphoid follicles reported in this study was relative, but not absolute, as some tetramer-staining cells were detected in those regions. Evaluation of chemokine receptor expression, particularly CXCR5, on those HIV-1-specific cells that were detected within lymphoid follicles and comparison to the chemokine receptor expression of tetramer-staining cells outside of follicles could provide important insight into the mechanisms that might be involved and suggest potential strategies to redirect CTL into follicles. Other pathogens that infect cells that normally reside within lymphoid follicles may exploit the immune privilege of lymphoid follicles as well. Indeed, we previously reported that in asymptomatic individuals coinfected with Kaposi's sarcoma herpes virus (KSHV) and HIV-1, KSHV latency-associated nuclear Ag is found primarily in lymphoid follicles (58), indicating that KSHV also colonizes this niche. Investigation of the relative distribution of other chronic pathogens and pathogen-specific CTL within lymphoid tissues is critical to evaluate the hypothesis that

multiple pathogens exploit the immune privilege of lymphoid follicles. Ultimately, a better understanding of the mechanisms that underlie the exclusion of CTL from lymphoid follicles as well as the possible role of lymphoid follicles in other diseases could lead to novel immunotherapeutic strategies to redirect CTL to lymphoid follicles and improve control of HIV-1 and many other seemingly intractable chronic infections.

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## Disclosures

The authors have no financial conflict of interest.

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